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14. ABSTRACT

Successful approaches to cancer immunotherapy require an understanding of the immune components in the microenvironment that enhance or inhibit tumor development and growth. Evidence is mounting that distinct T cell subsets contribute in positive or negative ways to these processes. An emerging area of interest relates to mechanisms by which sensing of metabolic cues regulates T cell fate and the character of the immune response. Our recent research suggested that an important metabolic regulator, hypoxia-inducible factor (HIF) -1, is a critical regulator of the balance between regulatory T cells (Treg) and Th17 cells, both of which play a role in inflammatory carcinogenesis and tumor immunity. These findings suggest that HIF-1 is likely to play an important role in cancer potentially through boosting tumor promoting T cells (Th17). Thus, pharmacologic inhibition of HIF-1 in combination with therapies that target regulatory T cells may inhibit the two T cell types capable of promoting tumors or protecting them from eradication by the immune system. This project explores the efficacy by targeting the HIF-1 and Tregs in mouse cancer model. These experiments should provide new insights into the role of regulatory T cells and Th17 cells in cancer, and help us to design combination immunotherapy strategies that will be more successful in treating patients.

15. SUBJECT TERMS
Regulatory T cells; Th17; Immunotherapy; HIF-1

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Introduction:

T cells play critical roles in immune response. An important metabolic regulator, Hypoxia-inducible factor (HIF)-1, can determine the balance between regulatory T cells and Th17 cells(1, 2), both of which play a role in inflammatory carcinogenesis and tumor immunity. We have set out to determine if HIF-1's potential for shaping the T cell response can affect the immune response to aggressive prostate cancer. We will also test the efficacy of HIF-1 inhibiting compounds as potential immune-modulators of the anti-tumor immune response.

Key Words:

Hypoxia-inducible factor (HIF)-1; regulatory T cells (Treg); Th17 cells; Immunotherapy

Accomplishments:

What were the major goals of this projects?

The major goals of this project include the three aims as follows:

I. **Analyzing the consequences of genetic HIF-1 modulation on prostate-infiltrating lymphocytes and tumor progression in the Pro-HA x TRAMP Mouse Model;**

II. **Development of an in vivo HIF-1 reporter assay and correlation with Th17 cytokine production during tumor progression in the mice model;**

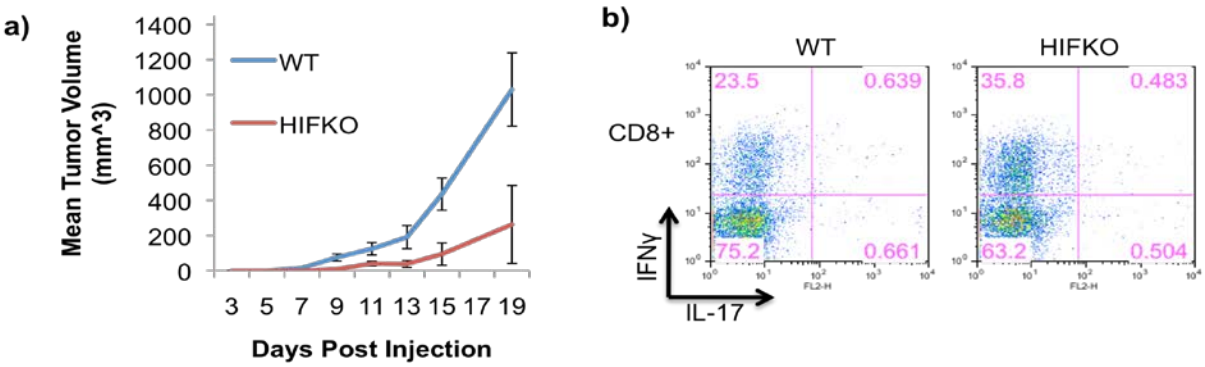
III. **Determine the efficacy of HIF-1 inhibitors in combinational modulation of the anti-tumor immune response, inhibition of tumor progression and augmentation of prostate cancer vaccines in vivo.**

What was accomplished under these goals?

For this reporting period, we, in collaboration with Dr. Chuck Drake's laboratory at Hopkins are actively developing a highly physiologically relevant mouse model for prostate cancer. While we intend to use the Pro-HA TRAMP model of prostate cancer(3), another model, more closely resembling the human disease has arisen. It has been reported that Myc-driven murine prostate cancer shares the same molecular features as human prostate tumors (4). Additionally, this model closely mimics the kinetics of tumor development in humans. We are expecting to use this mouse model in the 2nd year of DoD grant support. While we are waiting for this prostate cancer mouse

model to become available for the proposed experiments, we have explored the efficacy of HIF-1 inhibitors in combination with Treg depletion in another aggressive tumor model, the B16 model of melanoma (Aim 3). The results are summarized as follows:

HIF-1 deficiency and the anti-tumor immune response: While we are generating transgenic mouse colonies needed for the prostate cancer experiments, we have concentrated on exploring the role of HIF-1 in progressing tumors in an alternative model - the implantable B16 melanoma. These studies have yielded both considerable insight into HIF-1's role and enthusiasm for pursuing HIF-1 blocking agents as potent immunotherapies. Initially, we were encouraged by the observation that subcutaneous B16 tumors grew substantially slower in mice lacking HIF-1 in their T cells relative to wild type controls (Fig. 1A). As predicted by our earlier work, these



knockout mice displayed heightened frequencies of Foxp3+ cells – an observation made curious by the resoundingly pro-tumor effects of these cells (data not shown).

Figure 1. The effect of T cell-restricted HIF-1 ablation on tumor growth and the anti-tumor immune response. (a) WT and conditional HIF-1 knockout mice were injected subcutaneously (s.c.) with 1x10^5 B16 melanoma cells and tumor growth was monitored. The mean tumor volumes for each group are shown +/- SEM. (b) Tumor-infiltrating leukocytes were recovered and production of proinflammatory cytokines were assessed by flow cytometry.

As previously reported, Th17 responses to B16 challenge were muted in all groups(5). Surprisingly, CD8+ T cells recovered from the tumors of HIF-1 knockout mice proved more likely than wild type cells to produce the proinflammatory, and tumoricidal cytokine, IFNγ (Fig. 1B). Production of this cytokine in the CD4+ T cell compartment was not as dramatically altered by HIF- 1 deficiency (data not shown). In addition to controlling tumor growth, HIF-1 deficient T cells could also limit the spread of tumor cells in a metastasis tumor model. Intravenous injection of wild type mice with B16 cells results in the accumulation of lung metastases. Conditional HIF-1 knockout mice, in contrast, were resistant to the buildup of lung microtumors (Fig 2 a-c), and as seen in the s.c. tumor model, had an enhanced population of CD8+ cells making IFNγ (Fig. 2d).

These results suggest that HIF-1 negatively impacts the generation of a tumor-killing CD8+T cell subset. In line with this, RNASeq analysis of CD8+ T cells lacking HIF-1 revealed

that in the absence of this important metabolic regulator, CD8+ T cells take on an unusual memory-like phenotype (data not shown), which in agreement with recent studies (6), are more effective killers of tumor cells than conventional effector CD8+ T cells. Encouraged by these results, we tested whether HIF-1 inhibition could be an effective anti-cancer treatment strategy.

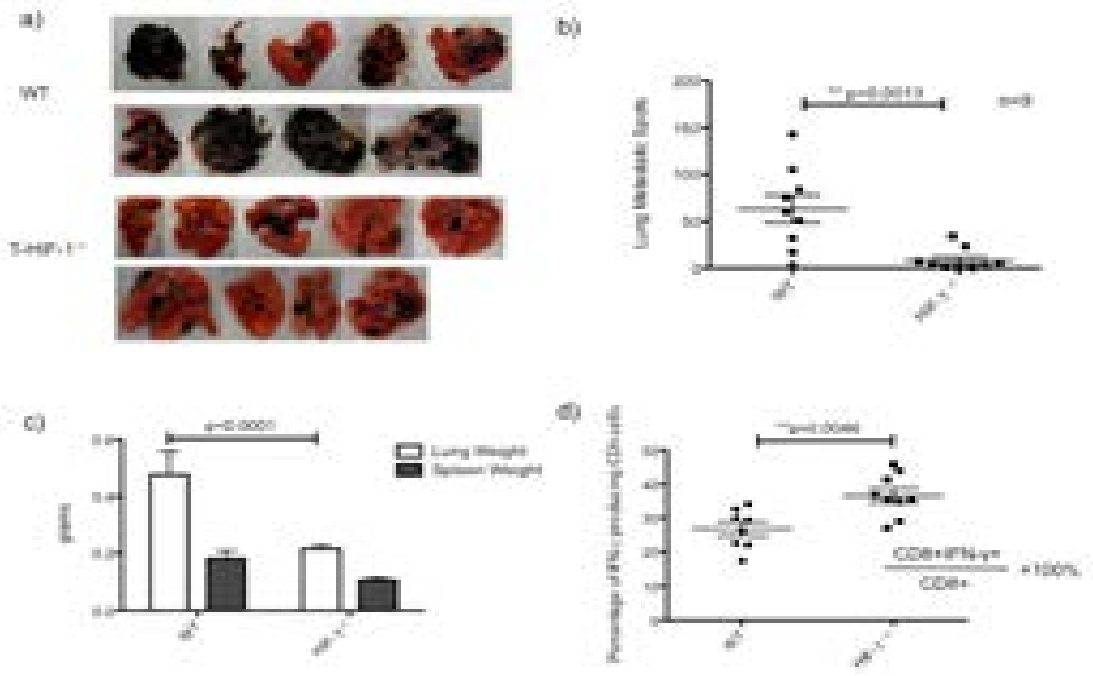


Figure 2. Mice lacking HIF-1 in T cells control the spread of tumors and harbor more IFN!-producing CD8+ T cells than wild type mice a) photographs of the lungs; b) the numbers of lung tumor nodules; c) the weight of the lungs and the spleens; d) the percentage of IFN-γ producing CD8 cells in the spleens from the WT and T- HIF-1KO mice injected i.v. with 1x10^5 B16 melanoma cells.

To this end, mice implanted with s.c. B16 tumors were treated daily with i.p. injections of vehicle (PBS) or HIF-1 inhibitors (ACF and DIG, "A/D"). As predicted by our prior results, HIF-1 targeting dramatically slowed the growth of tumors (Fig. 3) and mirrored the effects that genetic HIF-1 ablation had on CD8+ T cell differentiation (data not shown).

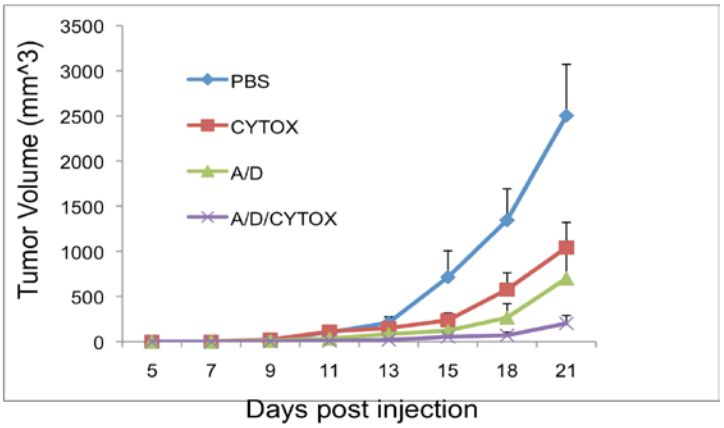


Figure 3. Treatment of mice with the HIF-1 inhibitors Acriflavine and Digoxin (A/D) stunted the s.c. growth of B16 melanoma cells in C57BL6 mice, as did low dose (75mg/kg) cytoxan (Cytox) – a Treg-depleting regiment. Combining HIF-1 inhibitors and Cytox treatment (A/D/Cytox) treatment (75mg/kg) almost completely prevented tumor growth.

Since targeting HIF-1 can enhance Foxp3+ T cell accumulation, as we had seen in our HIF-1 restricted knockout mice as well as HIF-1 inhibitor-treated, ETBF-afflicted mice, we suspected that a build-up of these tumor-abetting, immunity suppressing cells might be dampening the anti-tumor effect of HIF-1 targeting. Indeed, inhibitor treated mice did show elevated Foxp3+ T cell frequencies (data not shown). This compelled us to test the simultaneous inhibition of HIF-1 with known Treg depleting agents as a combinational treatment. Indeed, coupling low dose cytoxan (cyclophosphamide; 75mg/kg) with HIF-1 inhibitors reduced tumor growth to a greater extent than that seen with either monotherapy (Fig. 3). These results suggest that HIF-1 targeting therapies, especially along with Treg-antagonism, offer much promise as immune- modulating interventions to combat tumors. Future work will determine whether tumors of the prostate and other tissues are as susceptible to this novel treatment strategy. A manuscript describing these findings is currently being prepared for submission.

Summary and Key Accomplishments:

Thus far we have 1) established an effective dosing regimen for the use of HIF-1 inhibitors in tumor models in vivo; 2) demonstrated the added efficacy of combining HIF-1 targeting treatment with Treg depletion agents; 3) uncovered and characterized an unexpected role for HIF-1 in modulating the immune response to tumors (i.e. shaping CD8+ T cell phenotypes); and 4) improved our protocols for the isolation of tumor-infiltrating leukocytes.

What opportunities for training and professional development has the project provided?

This project has necessitated the learning and refinement of a widely used model for tumor growth in mice (i.e. the B16 melanoma model). We have multiple individuals and collaborators affiliated with the research team. These same individuals are in the process of learning the colony management skills as well as the experimental skills needed for the proposed prostate cancer models. We have also become familiar with the sample processing protocols associated with RNASeq analysis of immune cells that will serve us well in future experiments.

How were the results disseminated to communities of interest?

Nothing to report at this time.

What do you plan to do during the next reporting period to accomplish the goals?

In close collaboration with Dr. Chuck Drake, a prostate expert at Cancer center here, we will continue to work towards the establishment of the prostate cancer mouse models mentioned above. We are expecting to the pertinent strains be available in next few months. We will immediately repeat the above-discussed experiments in the prostate setting. We are expecting

that HIF1 inhibitors will slow prostate tumor growth. We will further dissect the gene profile changes among T cells isolated from the tumor tissue (TILs) of mice treated with HIF-1 inhibitors alone or in combination with other immunotherapeutic.

IMPACT: Nothing to Report at this time

CHANGES/PROBLEMS: Nothing to Report at this time

PRODUCTS: Nothing to Report at this time

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name: Fan Pan
Role: PI
Contribution to Project: Dr. Pan has designed the proposed experiments, operates the lab in which they will be carried out, drafted the proposal and report, and analyzed data.
Other Funding Support: NIH

Name: Joe Barbi
Role: Postdoc Research Fellow
Contribution to Project: Dr. Barbi assisted with the design of experiments, carried out experiments, collected results and analyzed data.
Other Funding Support: Crohn's and Colitis Foundation of America Research Fellowship (2012-2015)

Has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period? No

What other organizations have been involved as partners? No

SPECIAL REPORTING REQUIREMENTS: None

Appendices: None

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